Immunohistochemistry in Historical Perspective: Knowing the Past to Understand the Present

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Chapter 2

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Carlos Ortiz Hidalgo

Abstract

Immunohistochemistry is an extraordinary and extensively used technique whereby antibodies are used to detect antigens in cells within a tissue section. It has numerous applications in medicine, particularly in cancer diagnosis. It was Albert Hewett Coons, Hugh J Creech, Norman Jones, and Ernst Berliner who conceptualized and first implemented the procedure of immunofluorescence in 1941. They used fluorescein isothiocyanate (FITC)-labelled antibodies to localize pneumococcal antigens in infected tissues. Since then, with improvement and development of protein conjugation, enzyme labels have been introduced, such as peroxidase and alkaline phosphatase. The history of immunohistochemistry (IHC) combines physiology, immunology, biochemistry, and the work of various Nobel Prize laureates. From von Behring who was awarded de first Nobel Prize in 1901 for his work on serum therapy to the 1984 Nobel Prize for the discovery of monoclonal antibodies by Milstein, Kohler, and Jerne, IHC is a story of cooperation and collaboration which led to the development of this magnificent technique that is used daily in anatomical pathology laboratories worldwide.

Key words Immunohistochemistry, History, Antibodies, Hybridoma

1 Introduction

Italian-American Professor Juan Rosai once wrote, "In my opinion, there is no single technique in the vast medical arsenal that provides so much information, so quickly and for so little cost, than the hematoxylin and eosin technique (H&E)" [1, 2]. And although it is undoubtedly the most important method in basic histology and to establish the diagnosis in histopathology, in the molecular era, H&E stain has certain limitations [3].

Immunohistochemistry (IHC) is an experimental and diagnostic method that has revolutionized the practice of cytology, histology, and pathology that identifies a variety of molecules within cells of tissue sections utilizing an antigen-specific antibody on routine histological material through the conventional optical microscope [4]. IHC is widely used in the diagnosis of abnormal cancerous cells

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thorough localizing specific molecular markers that are characteristic of particular cellular events, such as cellular differentiation, proliferation or cell death (apoptosis). It is also used in basic research to understand the distribution and localization of diverse biomarkers and differentially expressed proteins in different parts of a biological tissue [4]. This histopathological technique, which began at the end of the last century, uses antibodies that allow the identification of specific cellular antigens through antigen–antibody reactions and subsequently revealed enzymatically. It is based on the great specificity and high affinity of antibodies to identify molecules and join them. The antibodies that are used in immunohistochemistry are the IgG type produced by plasma cells. Due to the number of antibodies available and to the standardization of its protocol, immunohistochemistry has become a simple and efficient laboratory method [5].

2 History of Immunohistochemistry

The development immunochemistry derived from the practical problems with the diphtheria antiserum assay, introduced into medical practice during the years 1888–1894 [6]. Émile Roux (1853–1933) and Alexandre E. J. Yersin (1863–1943), while working at the Pasteur Institute in Paris, showed that filtrates of diphtheria cultures that had no bacilli contained a substance, which they called "toxin," that produced all the symptoms of diphtheria when injected into animals (Fig. 1a, b) [7]. From these cultures of diphtheria bacilli, Ludwig Brieger (1849–1919) and Carl Fraenkel (1861–1915) prepared a substance, which they called *toxalbumin*, that immunized guinea pigs against diphtheria when injected. Later on, Emil von Behring (1854–1917) together with the Japanese bacteriologist Shibasaburo Kitasato (1852–1931), while working at the Pharmacological Institute of the University of Bonn, confirmed that if an attenuated form of diphtheria bacteria was injected into a guinea pig, the animal would produce "anti-toxins" in the serum, and these anti-toxins, in turn, could be used as therapeutic agents to prevent and fight the disease (Fig. 1c, d). This great discovery was soon confirmed and successfully used by other workers. Behring was awarded the first Nobel Prize in Physiology and Medicine in 1901 "for his work on serum therapy, especially its application against diphtheria, by which he has opened a new road in the domain of medical science and thereby placed in the hands of the physician a victorious weapon against illness and deaths" [8]. There were 46 nominees for the first Nobel Prize in Medicine in 1901, which included Kitasato, Ehrlich, Golgi, Koch, and Ramón y Cajal, amongst others, but Behring was nominated officially and became the first winner of the Nobel Prize in Physiology or Medicine; Kitasato never appeared in the list of nominees again [9].

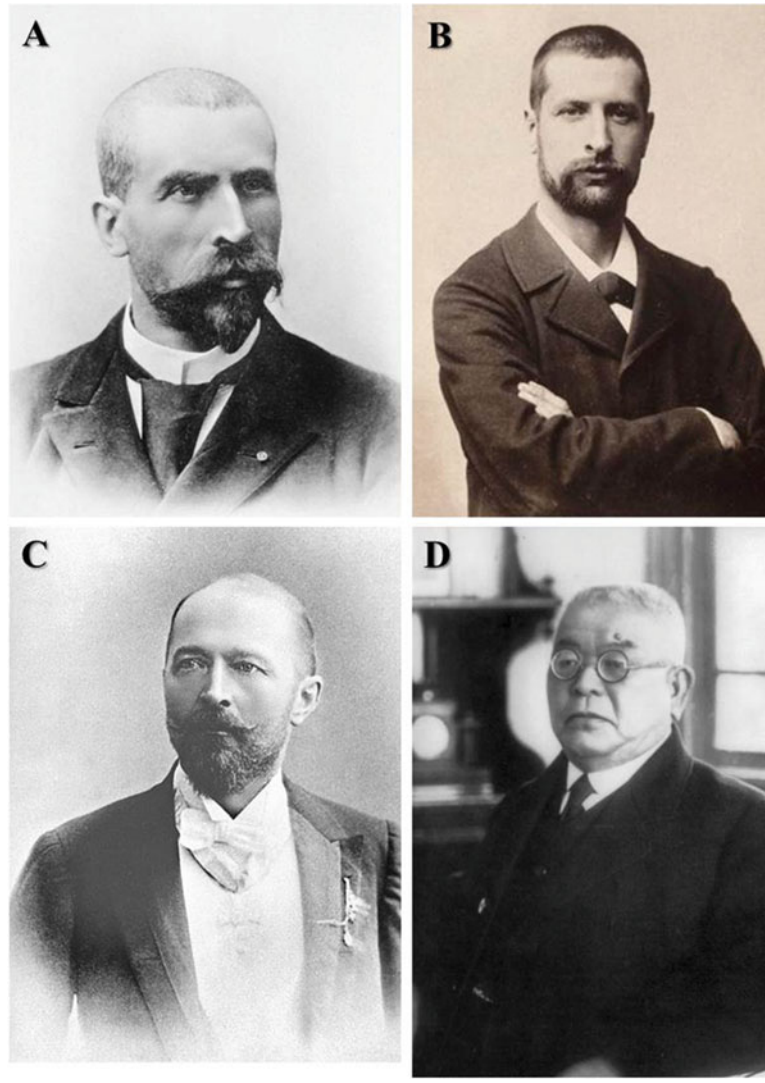

Fig. 1 (**a**) Pierre Paul Émile Roux (1853 Confolens, France-1933 Paris, France), (**b**) Alexandre Emile John Yersin (1863 Lavaux, Cantón de Vaud, Switzerland-1943 Nha Trang, Vietnam), (**c**) Emil Adolf von Behring (1854 Hansdorf, Germany-1917 Marburg, Germany), (**d**) Baron Shibasaburo Kitasato (1852 Okuni, Japan-1931 Nakanocho, Japan)

Today, we know that antibodies (the Behring antitoxins) are produced by the immune system in response to foreign molecules, but the nature of these substances was not known at that time. Together with Paul Ehrlich (1854–1915), who was one of the most outstanding figures in the world of science, Behring established a laboratory in Berlin, where they made the first immunocytochemistry research that contributed to the clarification of the composition of the so-called Behring antitoxins [10]. In 1896, Ehrlich was appointed the first director of the *Institut für Serumforschung*

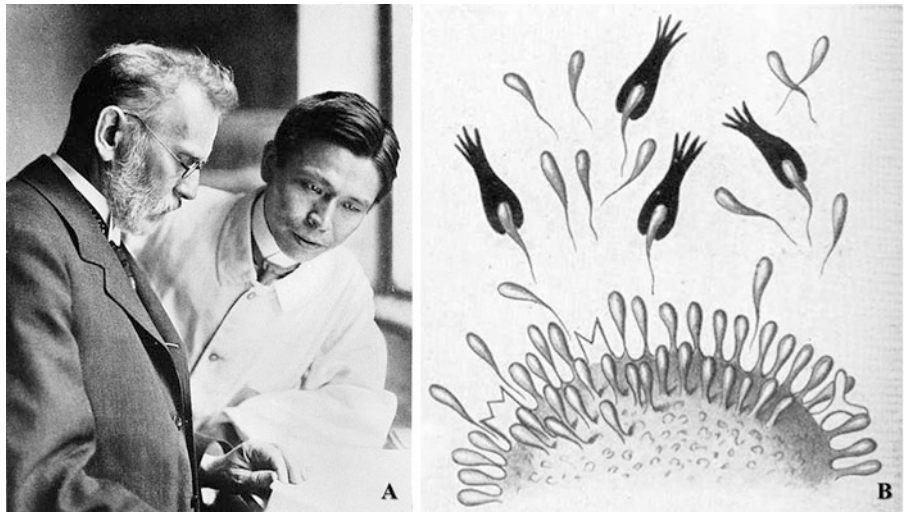

Fig. 2 (**a**) Paul Ehrlich (1854 Strehlen, Silesia, Prussia [now Strzelin, Poland]–1915 Hamburg, Germany) and Sahachiro Hata (1873 Tsumo Village, Japan–1938 Tokyo, Japan) at the Institute of Serum Research and Serum Testing, today *Paul-Ehrlich-Institut.* (**b**) Diagram illustrating Ehrlich's side-chain model of immunity. (Adapted from: Ehrlich P. Croonian lecture: on immunity with special reference to cell life. Proc Royal Soc Lond 1900: 66: 424–448)

und Serumprüfung (Institute of Serum Research and Serum Testing, today *Paul-Ehrlich-Institut*) southwest of Berlin, and with his assistant Sahachiro Hata (1873–1938), characterized and quantified the toxin–antitoxin reaction and described the selectivity of these "antitoxins," also predicting its possible use as a "magic bullet" (*zauberkugel*) to act specifically against a particular pathogen without causing damage to the cells of the host or in the treatment of tumors (Fig. 2) [10]. Ehrlich attributed the specific activities of antigen and antibody to definite chemical structures and represented these structures with the "lock and key" diagram. The problem was to find some concrete "lock and key" structures. Ehrlich received jointly with Élie Metchnikoff (1845–1916) the Nobel Prize for Physiology or Medicine in 1908. In his Nobel Prize lecture Ehrlich mentioned, "... The first advance in this complicated field came about, as so frequently happens, in a roundabout way. After Behring's renowned discovery of the antitoxins I had set myself the task of penetrating further into the mysterious nature of this process, and after long labours I have succeeded in finding the key to it ..." [11].

Around 1913, studies conducted by Oswald T. Avery (1877–1955) at the Rockefeller Institute Hospital in New York City, demonstrated that human immune systems responded to different strains of pneumococcus [12]. Along with the American bacteriologist Alphonse R. Dochez (1882–1964), Avery confirmed the correlation between virulence and the presence of a bacterial capsule, which protects the bacteria against ingestion by other

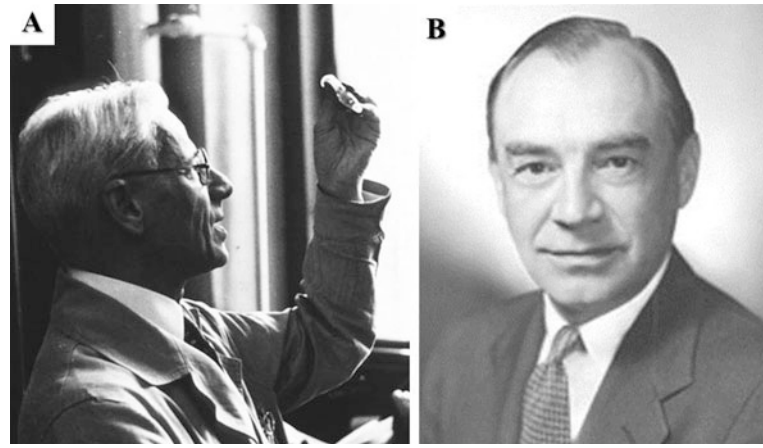

Fig. 3 (**a**) Michael Heidelberger (1888 New York City–1991 New York City) and (**b**) Albert Hewett Coons (1912 Gloversville, New York–1978 Brookline, Massachusetts)

microorganisms, and discovered that differences in surface carbohydrates (polysaccharides) characterize strains of pneumococcus and their virulence. They also found that the polysaccharide could stimulate an immune response and were the first to establish that a substance other than a protein could do so. The evidence that the polysaccharide composition of a bacterium influences its virulence and its immunological specificity made them realize that these characteristics could be analyzed biochemically, thus contributing to the development of immunochemistry [12].

By the late 1920s, the chemical nature of antibodies was not yet known. In 1923; one of Avery's pupils, Michael Heidelberger (1888–1991), who is considered one of the founders of immunohistochemistry, determined that the antibodies made against pneumococcal polysaccharides were proteins [13] (Fig. 3a). In experiments conducted during the early 1930s, Heidelberger and his postdoctoral fellows Forrest E. Kendall (1899–1987) (later Professor Emeritus of Biochemistry at Columbia University) and Elvin A. Kabat (Kabatchnick) (1914–2000) were able to measure the amount of antibody in washed antigen–antibody precipitates and developed the first antigen–antibody complex identified microscopically by means of attaching a purple azo dye to the antigen. When specific antibody was added, the color antigen–antibody complex precipitated. It was then possible to measure antibodies, which verified for the first time that circulating antibodies were modified serum globulins and not substances of unknown composition merely associated with the globulins of serum. Heidelberger, who was also a talented musician and linguist as well as a very close friend of the 1959 Nobel Prize winner Severo Ochoa de Albornoz, worked in his laboratory until his death at the age of 103 [14].

The biochemical antigen–antibody complex formation was described by Professor John Richardson Marrack, MA, MD (1886–1975) in 1934 [15]. Marrack was first a lecturer in chemical pathology at Cambridge, later on a lecturer at the Hospital Medical College in London, and remained at the Hale Clinical Laboratories at London Hospital until his retirement in 1952 [16]. After some preliminary work on the physical chemistry of proteins, Marrack began a series of studies on the antigen–antibody reaction that remain his main academic interest. His work on immunochemistry began in the late 1920s and concentrated on the antigen–antibody reaction [15]. His research showed that the precipitate formed by diphtheria toxin and antitoxin was mostly antitoxin, and the antitoxin was composed of serum globulin and showed that increasing amounts of antigen resulted in an increase in the proportion of antigen in the complexes. Similar findings had been made by Heidelberger and Kendall in New York [17]. Marrack proposed that each antibody could bind with two antigenic polyvalent molecules and produce a precipitating complex in the form of a network. He incorporated a red dye to the antibodies by joining them to an R-salt with tetrazotized benzidine, demonstrating that the specific antigens dyed or marked red. Marrack wrote, in 1934, a book entitled "*Chemistry of Antigens and Antibodies*" that summed up the existing knowledge of the problem of the antigen–antibody reaction. This text is considered to be one of the most influential books in immunology [18, 19]. With all the studies described above, the arena was set for the development of immunohistochemistry [20].

The labeling method of antibodies with fluorescein was developed in 1941 by Albert Hewett Coons (1912–1978), professor in the Department of Bacteriology and Immunology at Harvard Medical School and a member of the National Academy of Sciences (Fig. 3b). Coons began a major revolution in immunology and cell biology, developing the immunofluorescent technique for labeling specific antibodies with fluorescent dyes, thus permitting the detection of antibodies, antigens, and virtually any antigenic protein in cells and tissues [5, 6].

Coons, born in Gloversville, New York, on June 28, 1912, began his research with the study of patients with injuries caused by rheumatic fever, and he recalls: "At the end of my internship I had a six months' gap before my next appointment as an assistant resident, and I was lucky enough to be able to spend them in Berlin ... I had an entrée into the Pathological Institute at the Charité Krankenhaus ... I spent my mornings watching autopsies, and my afternoons wandering around the city talking to people in cafes and trying to improve my halting German. In strange cities, visitors have many hours alone. I found myself walking in the streets or sitting in my room reading or brooding. One afternoon I was thinking about rheumatic fever and about the Aschoff nodule, the

microscopic lesion characteristic of it. It was at that time, and I think probably in many circles still is, thought to be the result of a local hypersensitivity reaction involving components of the group A hemolytic streptococcus and circulating antibodies or hypersensitive cells. It struck me that this theory had never been tested and indeed could not be tested without the demonstration of antibody or antigen, preferably both, in the local lesions. I considered that it might be easier to find the antigen than the antibody, for a start anyway, and that what was required was a visible microprecipitate. The notion of labeling an antibody molecule with a visible label was perfectly obvious in such a context. However, when I tried this notion on my friend, Apitz, he was not enthusiastic. I think he thought it was not feasible and indeed, in the terms in which I initially thought of it, as a colored molecule, it wasn't ..." [21].

Coons began his career with the idea of becoming a clinician and perhaps a teacher in a medical school. His interest in research and immunology was enthused by the influence of Hans Zinsser (1878–1940), who was Professor of Bacteriology and Immunology at Harvard Medical School, and by George R. Minot (1885–1950), who shared the 1934 the Nobel Prize in Medicine with George H. Whipple and William P. Murphy, for their pioneering work on pernicious anemia. Coons took Dr. Zinsser's course in immunology, which stimulated him to work during the summer of 1935 with John Enders (1897–1985), then an assistant professor in the department (and a Nobel Prize Laureate in Medicine in 1954 for the discovery of the ability of poliomyelitis viruses to grow in cultures of various types of tissue) [6]. Enders advice to Coons was that labeled antibodies might not answer the rheumatic fever question, but they might provide a general procedure for locating antigens in tissues and cells and clearly would have application to countless problems [21]. In Enders's lab, Coons learned how to handle different species of antibodies and the precipitin reaction used to visualize antigen–antibody complexes in a test tube. The antibody solutions, Coons noticed, were still active in agglutination reactions, and it was easy to show that the clumped organisms were specifically reddened by the colored antibody. However, when he repeated these experiments, he found that under the microscope the organisms were only faintly pink, so there was little hope of being able to find small amounts of antigen in tissues with a color of that intensity. He thought of using fluorescence instead. In 1941, Coons and his coworkers, Hugh J. Creech, Norman Jones, and Ernst Berliner, demonstrated that it was possible to localize antigens in tissue sections using fluorescein isothiocyanate (FITC)-labeled antibodies to localize pneumococcal antigens in infected tissues and visualized by ultraviolet light (fluorescence microscopy). Fluorescein was chosen because it fluoresces with a brilliant apple green color, which is not seen in any normal tissues [5, 6].

Using the fluorescent anti-pneumococcal antibody conjugate, they were able to find bacterial polysaccharides in the macrophages of mice injected intravenously with large numbers of pneumococci, thus succeeding in demonstrating the possibility of putting fluorescent tags on antibodies and using them to localize foreign antigens in host tissues. Coons stated that the fluorescent technique "... was like putting tails of colors to the antibodies!" His initial results were described in two papers published in the "Proceedings of the Society for Experimental Biology and Medicine" and in the "Journal of Immunology" in 1941 and 1942, respectively [22, 23]. Coons was awarded the Albert Lasker Prize in 1959, the Paul Ehrlich Prize in 1961, the Passano Award in 1962, the Gairdner Foundation Annual Award in 1963 and the Emil von Behring Prize in 1966. In 1960, Coons was nominated for the Nobel Prize for his studies on the fluorescent antibody labeling method but was not considered "prize-worthy"! [24]. He died of a heart attack on September 30, 1978, at the age of 66.

The immunofluorescence method was successfully used as a powerful research tool during the next 25 years but, to a certain extent, was of limited utility, particularly for surgical pathology diagnosis, since fresh tissue and a fluorescence microscope is required. Additionally, fluorescein fades as the tissue section is viewed (even though nonfading fluorescent markers are now available), and immunofluorescence does not offer the best morphology on tissue sections, especially for diagnostic purposes; therefore, labels other than fluorescein isothiocyanate were sought [5, 6]. It was during the 1960s that researchers tried to attach enzymes to antibodies (enzyme-labeled antibodies), so they could be visualized without the use of florescence microscopy. Several enzymes, including horseradish peroxidase, glucose oxidase and alkaline phosphatase, were evaluated along with a number of different substrate systems to determine the site of location of the enzyme-labeled antibody within tissue sections [5]. The use of enzymes as tracers for antibodies and antigens and the methods to label them led to the development of methods such as ELISA (enzyme-linked immunosorbent assay), Western blot, Southwestern blot, Southwestern histochemistry and enzyme in situ hybridization. The enzyme immunolabeling protocols had many advantages. They improved specificity by using more diluted primary antibodies, the labeling would not fade, and outstandingly, the sections could be seen with the use of a routine bright-field microscope. Various researchers addressed the problem. In 1966, Paul K. Nakane and G. Barry Pearce, while working at the University of Michigan, used acid phosphatase and horseradish peroxidase [6]. As Clive Taylor has shared, the final critical step in making immunohistological methods available to the biological researcher and surgical pathologist was taken in the 1970s using the immunoperoxidase technique, in which the antibodies are visualized via a peroxidase-catalyzed

reaction [5]. The most widely used substrate, diaminobenzidine, polymerizes in the presence of peroxidase and hydrogen peroxide to form an insoluble brown polymer, which is deposited at the site of the antigen–antibody reaction [25]. Peroxidase can be detected by reacting with hydrogen peroxide in the presence of an electron donor, because it forms colored oxidation products. 3-3 diaminobecidine (DBA) is generally used as an electron donor, a technique that was pioneered by Morris J. Karnovsky (1926–2018) and Richard C. Graham of the Department of Pathology at Harvard University [6]. DAB gives a brown/amber color to the reaction and one can add nickel salts that will make DAB precipitate to be blue or black. The following discoveries of the unlabeled antibody–peroxidase–anti-peroxidase (PAP) method by the German born biochemist Ludwig Amadeus Sternberger (1921-) and colleagues in the late 1970s and the alkaline phosphatase–anti-alkaline phosphatase (APAAP) method by the British pathologist David York Mason (1941–2008), significantly expanded the application of the immunohistochemistry technique [5, 26]. Mason along with Taylor were the ones who first showed the diagnostic potential of immunoperoxidase techniques for studying human tissue samples, particularly for the diagnosis of lymphomas and leukemias. Mason was one of the most enthusiastic promoters and organizers of the Human Leucocyte Differentiation Antigen (HLDA) Workshops that contributed to monoclonal antibody nomenclature that led to the introduction of the "CD" clusters of differentiation system used worldwide today [27]. For a long time, the use of soluble peroxidase–anti-peroxidase complexes has been incorporated into many laboratories as a routine practice for detecting antigens in tissue sections. Currently, however, this method has been replaced by the avidin, biotin and streptavidin methods [5, 28]. The discovery of antigen retrieval methods by Huang et al. in 1976 and also the systems of secondary antibody detection (e.g., the avidin–biotin–peroxidase complex [ABC] and the labeled streptavidin–biotin complex [LSAB]) by Hsu et al. in 1981 allowed immunohistochemistry to be used in fresh specimens as well as in fixed tissues, which further increased the applicability of the technique, particularly in diagnostic pathology routines [29].

3 The Hybridoma Era

The development of ultracentrifugation and electrophoresis by the Swedish scientists and Nobel Laureates Theodore Svedberg (1884–1971) and Arne W. K. Tselius (1902–1971), respectively, led to the physical characterization of antibodies and γ-globulins [6]. The chemical structure of antibodies was independently discovered by Rodney R. Porter (1917–1985) and Gerald M. Edelman (1929–2014) who were jointly awarded the Nobel

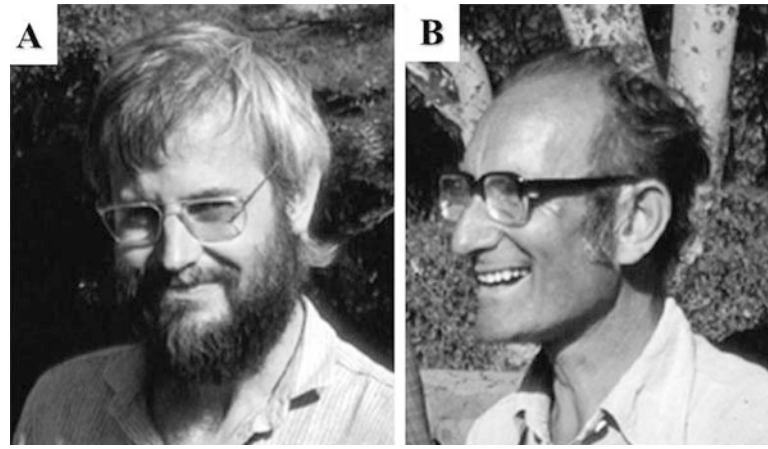

Fig. 4 (**a**) Georges Jean Franz Köhler (1946 Munich–1995 Freiburg) shared the Nobel Prize in 1984 with (**b**) Cesar Milstein (1927 Bahía Blanca, Argentina–2002 Cambridge, UK) for their work in the production of mAbs by hybridizing mutant myeloma cells with antibody-producing B-cells (hybridoma technique). The production of mAbs revolutionized immunological research

Prize in Physiology or Medicine in 1972 [30]. Multiple myeloma immunoglobulins released by neoplastic plasma cells, Bence-Jones proteins and monoclonal macroglobulins from patients and mice, played a central role in the elucidation of normal immunoglobulin structure, genetics synthesis and metabolism.

In 1975, the 28-year-old German postdoctoral fellow George J. F. Köhler (1946–1995) and Argentinian born biochemist César Milstein (1927–2002), while working at the Medical Research Council Laboratory of Molecular Biology in Cambridge, UK (one of the world's leading biomedical research institutes), published an elegant system of obtaining pure antibodies of known specificities in large amounts [31] (Fig. 4). "Such cultures could be valuable for medical and industrial use," read the final sentence of an immunological paper published in the August 7, 1975, issue of Nature [31]. For this discovery, 9 years later, Milstein and Köhler shared the Nobel Prize in Physiology or Medicine together with Niels K. Jerne (1911–1994) (then working at the Basel Institute for Immunology in Switzerland) for "theories concerning the specificity in development and control of the immune system and discovery of the principle for production of monoclonal antibodies," which is by far one of the greatest biological advances of the twentieth century [32]. The technique of monoclonal antibody production revolutionized immunology and had major positive consequences for biology and medicine [33].

It was in the 1960s that the American molecular biologist Michael Potter (1924–2013), while working at the National Cancer Institute, Bethesda, Maryland, made the discovery that an injection of mineral oil into the peritoneal cavity of the BALB/c

albino laboratory-bred strain of the house mice induced the growth of myeloma cells easily and indefinitely. Potter and his colleagues established a collection of myeloma cells lines for distribution to researchers around the world [34]. One month after the Nobel Prize announcement of Köhler, Milstein and Jerne, Potter received the Lasker Award for basic medical research (shared with Milstein and Köhler), which is considered second in importance only to the Nobel Prize [34]. Using the technique developed by Potter, Milstein's group established a culture of rapidly proliferating tumor cells that produced immunoglobulins. In Köhler and Milstein's method, a mouse is immunized repeatedly with the desired antigen and the spleen is removed, which contains proliferating B-lymphocytes. The B-cells are immortalized by fusion (by chemical or viral induced methods) with a nonsecretory myeloma plasma cell, and the resulting hybridoma can then secrete large quantities of the antibody encoded by its B-cell fusion partner. Köhler and Milstein demonstrated that plasma cells of virtually any desired specificity could be fused with a myeloma cell line; the result being unlimited amounts of homogeneous (monoclonal) antibodies carrying that specificity [33]. Monoclonal antibodies (mAbs) are monovalent antibodies that bind to the same epitope and are produced from a pure single B-lymphocyte culture line. In contrast, polyclonal antibodies bind to multiple epitopes and are generally made by diverse plasma cell lineages [35, 36]. Today, it is possible to produce mAbs that are highly specific for diverse human tissues using hybridoma technology [37]. mAbs have paved the way for a rapid growth in the use of immunohistochemistry in research and has had a major positive impact on the development of biochemistry, immunology, molecular biology and medicine [36]. They are also used to measure the presence of hormones and proteins in the blood and to treat cancer and other diseases by being chemically linked to drugs, radioisotopes, and staining materials. They are vital to the development of new vaccines for diseases, such as herpes, hepatitis, malaria, and human T-cell leukemia, and are also used in preparing biologic materials, including interferons, growth factors, and hormones [36].

Jerne, born in London but raised in Holland, was known as a great theoretician in modern immunology, responsible for establishing an essential framework for understanding antibody formation and diversity [32]. After Jerne's retirement, the German chemist Georg Friedrich (Fritz) Melchers (1936-), Professor of Immunology at the University of Basel, took over the Director position and recruited the young Köhler as a PhD student. Köhler's attention was drawn to the benefit of using malignant B-cell lines for mutation studies, as was being practiced in the laboratory of Milstein in Cambridge [32]. Köhler's doctoral research concentrated on estimating how many antibodies animals could make against beta-galactosidase, an enzyme produced by the gram-

negative bacteria *Escherichia coli*. He injected beta-galactosidase into laboratory animals, including rats, rabbits and mice, and analyzed the antibodies contained in their serum. This work demonstrated that animals could generate many different types of antibodies against a single site on a foreign protein. Köhler completed his doctorate in April 1974 with the great distinction "magna cum laude" and gained a postdoctoral fellowship from the European Molecular Biology Organization to work in the laboratory of Cesar Milstein at the Medical Research Council Laboratory of Molecular Biology in Cambridge, UK.

Milstein was born in Bahia Blanca, Argentina on October 8, 1927. He studied chemistry at the Universidad de Buenos Aires and obtain his doctoral degree in 1957 [38]. He was granted a fellowship from the British Council, which led him to join the biochemistry department at Darwin College, University of Cambridge, UK, to work under the British biochemist Malcolm Dixon (1899–1985) on the project on the mechanism of metal activation of the enzyme phosphoglucomutase. While working with Dixon, he met the English biochemist and molecular biologist Frederick Sanger (1918–2013), who twice received the Nobel Prize for Chemistry in 1958 and in 1980, and joined his group on a short-term Medical Research Council appointment. Milstein returned to Argentina for a period of 2 years to run his own research group, but due to political problems in Argentina, in 1936, he was forced to return to Cambridge to rejoin Sanger, who by then had been appointed Head of the Division of Protein Chemistry in the newly formed Laboratory of Molecular Biology of the Medical Research Council. Following his suggestion, he shifted his interests from enzymes to antibodies. Milstein gave a seminar on his cell fusion work at the Basel Institute in 1873, and it was then that Köhler expressed his desire to work with him at Cambridge. After several years of hard work, they presented their results on the method for generating large amounts of mAbs of a predefined specificity in a session that was attended by many prominent scientists of the day, including the South African born and 2002 Nobel Prize winner Sydney Brenner (1927–2019) who asked, "Can one make an antibody to anything? Could I make one to my mother-in-law?" [32].

4 Epilogue

As Brandtzaeg pointed out, "Immunostaining is a way to talk with cells". Immunohistochemistry allows us to identify amino acids and various proteins, different infectious agents and the histological origin of the cell and its functions [28]. Numerous new entities have been described, refined and redefined, classified and reclassified, as this technique has provided new insights. The revolutionary

discoveries of fluorescein-labeled antibodies by Coons and colleagues in the early 1970s and application of the "immunoperoxidase" method to formalin-fixed, paraffin-embedded tissues by Taylor and Mason in Oxford was a critical step in extending the use of the method into routine diagnosis in histopathology [5].

In 1951, the American immunologist Henry George Kunkel (1916–1983) from the Rockefeller Institute in New York City noted that myeloma plasma cells produced antibodies of just a single specificity, and Richard (Dick) Cotton (1940–2015), an Australian researcher, was instrumental in the development of techniques to produce Monoclonal Antibodies. Cotton showed that the fusion of two immunoglobulin-producing myeloma cell lines produced a hybrid cell line that secreted myeloma antibodies of both parental types. Although the specificity of the antibodies produced was not known, this study was important for future work, as it described a technique for the production of hybrid clones. Köhler and Milstein succeeded in making fusions of myeloma cell lines with B-cells to create hybridomas that could produce antibodies specific to known antigens and that were immortalized. These discoveries have revolutionized today's basic research and diagnostic pathology practice. There are few areas of biomedical research and diagnosis that have not benefit from the availability of Monoclonal Antibodies [38].

Since the pioneering work of Köhler and Milstein, there have been numerous applications and refinements to the application of mAbs. Immunohistochemistry, immunofluorescence, immunoprecipitation, immunoblot analysis, radial immunodiffusion, and rocket immunoelectrophoresis as well as the double immunodiffusion test created by Örjan Ouchterlony (1914–2004) are additional techniques dependent on the use of antibodies that have been applied widely in the biological sciences. Thanks to their specific binding and the possibility for investigators to select the target of choice, Monoclonal Antibodies have proven to be a vital research and analytical tool that has had a truly transformative effect on clinical and laboratory sciences [39].

In the molecular era, immunocytochemistry will continue to undoubtedly play an important role in biological research and histopathological diagnosis. As professor Gwen V. Childs stated in his brilliant article on history of immunohistochemistry, "... We will continue to put 'tail lights on antibodies that will illuminate antigens like pebbles in the moonlight,' guiding us home to truths about their location and dynamic properties ..." [6]. Immunohistochemistry is one of the most important laboratory tools for scientific research and as a complementary technique in the differential of surgical pathology diagnosis, particularly for tumors that are not determinable by conventional hematoxylin and eosin (H&E) analysis [40]. This great technique became known as the "brown revolution" of basic histology and pathology laboratories [41].

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